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SYNTHETIC L-∝-DIOLEYL LECITHIN IN ANTIGENS FOR THE SERO-DIAGNOSIS OF SYPHILIS: COMPARISON WITH OTHER LECITHINS*†

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The lecithin which is used in cardiolipin antigens for the sero-diagnosis of syphilis is extracted from beef heart tissue or egg yolk by the methods of Pangborn, Maltaner, Tompkins, Beecher, Thompson, and Flynn (1951). The product is actually a mixture of individual lecithins containing chiefly unsaturated but also some saturated fatty acids, the composition varying with the source and method of isolation. It is necessary, therefore, to control the preparation of the lecithin very carefully. Even so, its concentration in an antigen may have to be changed slightly from one lot to the next, necessitating careful standardization of the antigen. Because of these disadvantages, we have been testing various pure lecithins as substitutes for Pangborn lecithin in antigens for the Kolmer complement-fixation test and the VDRL microflocculation test. They include saturated L-α-(dimyristoyl)-lecithin, synthesized by Baer and his associates (Baer, 1953; Baer and Kates, 1950; Baer and Maurukas, 1952), and the unsaturated L-α-(dipalmitoleyl)-lecithin extracted from yeast by Hanahan and Jayko (1952), both of which have shown definite promise as antigen components (Tonks and Allen, 1953, 1955).

The latest pure lecithin to be studied is unsaturated L-α-(dioleyl)-lecithin, which was synthesized for the first time by Baer, Buchnea, and Newcombe (1956).‡ Samples were obtained from Dr. Baer and were tested in antigens prepared for the Kolmer and VDRL tests. The antigens were found to be reactive. Since this synthetic material is stable and can be measured by weighing, and since it is an individual lecithin whose purity can be determined by physical and chemical methods, it holds promise as a lecithin component in cardiolipin antigens. Its properties have been compared with those of the other lecithins.

Method of Study

The dioleyl lecithin, which is a firm wax, was weighed accurately and dissolved in absolute ethanol to give solutions containing 15 mg. per ml. Antigens of various compositions were prepared volumetrically from them and from similar solutions of cardiolipin and cholesterol. The cardiolipin was obtained commercially (Sylvana) and had been approved for use in antigens. The cholesterol solution contained 1·5 per cent. Pfanstiehl cholesterol (precipitated from alcohol for the Kline test).

VDRL Microflocculation Test.—Antigens were prepared containing 0·02 or 0·03 per cent. cardiolipin, varying amounts of the L-α-(dioleyl)-lecithin, and 0·9 per cent. cholesterol (Table I, overleaf). These antigens were compared with our regular VDRL antigen** (composition 0·03 per cent. cardiolipin, 0·232 per cent. Pangborn lecithin, and 0·9 per cent. cholesterol) in order to select one possessing standard sensitivity. The selected antigen, No. 8, was then compared with one containing saturated L-α-(dimyristoyl)-lecithin (composition 0·03 per cent. cardiolipin, 0·30 per cent. lecithin, 0·9 per cent. cholesterol), and another containing unsaturated L-α-(dipalmitoleyl)-lecithin (composition 0·03 per cent. cardiolipin, 0·475 per cent. lecithin, and 0·9 per cent. cholesterol). These antigens had previously been found to have the same sensitivity as the standard antigen (Tonks and Allen, 1953, 1955).

The stability of saline suspensions of Antigen No. 8 was determined for a 24-hr period. Serum specimens were tested with the suspensions immediately and 0·5, 3, 6, and 24 hrs after they were prepared (Table II, overleaf). Each serum had been divided into several portions and a different portion was tested each time after inactivation

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** Since carrying out this work, the lecithin content of our standard antigen has been changed in order to lower its sensitivity slightly. It now contains 0·03 per cent. cardiolipin, 0·22 per cent. Pangborn lecithin, and 0·9 per cent. cholesterol.

TABLE I
VDRL TEST
ANTIGENS CONTAINING BAER'S UNSATURATED L-α-(DIOLEYL)-LECITHIN COMPARED WITH STANDARD ANTIGEN

Antigen No.	Composition (g./100 ml.)			Ratio of Cardiolipin to Lecithin	Comparison with Standard Antigen	
	Cardiolipin	Lecithin	Cholesterol		Positive Sera	Negative Sera
6	0·03	0·15	0·9	1:5	Much weaker	Same
7	0·03	0·30	0·9	1:10	Weaker	Same
13	0·03	0·36	0·9	1:12	Weaker	Same
14	0·03	0·405	0·9	1:13·5	Slightly weaker	Same
8	0·03	0·45	0·9	1:15	Same	Very slightly rougher
15	0·03	0·525	0·9	1:17·5	Slightly stronger	Slightly rougher
12	0·02*	0·40	0·9	1:20	Stronger	Rougher

* The percentage of cardiolipin was lowered to 0·02 per cent. so that an antigen of ratio 1:20 could be prepared from the same stock lecithin solution.

TABLE II
VDRL TEST
STABILITY OF ANTIGEN SUSPENSIONS (IN DUPLICATE) CONTAINING L-α-(DIOLEYL)-LECITHIN

Serum No.	Readings* at				
	0 hr	½ hr	3 hrs	6 hrs	24 hrs
1	2, 2	2, 2	2+, 2+	2, 2	2, 2
2	SR, SR	SR, SR	R, R	R, R	SR, SR
3	4, 4	4, 4	4, 4	4, 4	4, 4
4	2+, 3	2+, 2+	2+, 2+	2+, 3	2+, 2+
5	–, –	–, –	–, –	–, –	–, –
6	1, 1–	1, 1	1+, 1+	1, 1+	1, 1

* R=rough negative; SR=slightly rough. The plus and minus signs indicate slight differences in the particle size. It is recognized that in diagnostic work, findings with the VDRL test should be reported as "Reactive", "Weakly Reactive" and "Nonreactive", as recommended by the authors.

at 56°C. The suspensions were kept at room temperature in the glass-stoppered bottles in which they were prepared.

In order to test the reproducibility and purity of the lecithin material, many samples were obtained from Drs. Baer and Buchnea. In all, 21 independent preparations were tested. With many of the earlier ones, precipitates formed in the antigens when the cardiolipin and lecithin were combined. It was found that the precipitates were due to the presence of traces of barium chloride in the lecithin which caused the formation of an insoluble salt of cardiolipin. After testing various methods of purifying the lecithin, a procedure was finally developed which resulted in a pure material giving no precipitation on mixing with cardiolipin. Two lots of the final product were tested.

The stock alcohol solutions of the lecithin were stored at room temperature in the dark. Two of them were tested for stability by preparing batches of antigen of the same composition on several occasions during a period of 12 months and testing them serologically. The complete antigen also was examined for stability over a period of 8 months when stored in the dark at room temperature. Since these tests were carried out with Antigen No. 8 (Table I), which was known to have the same sensitivity as regular VDRL antigen, the latter was used as a control for all stability studies.

The lecithin in its solid form was similarly tested for stability over a period of 6 months.

Kolmer Complement-Fixation Test.—Antigens were prepared containing 0·0175 per cent. cardiolipin, different amounts of L-α-(dioleyl)-lecithin, and 0·3 per cent. cholesterol (Table III). Comparisons were made with our standard Kolmer cardiolipin antigen of composition 0·0175 per cent. cardiolipin, 0·200 per cent. Pangborn lecithin, and 0·3 per cent. cholesterol (Allen and Mason, 1952). Antigen dilutions of 1:300 were found to be optimal for most preparations. Finally, the L-α-(dioleyl)-lecithin antigen selected as having standard sensitivity was compared serologically with one containing saturated L-α-(dimyristoyl)-lecithin (composition 0·0175 per cent. cardiolipin, 0·1225 per cent. lecithin, 0·3 per cent. cholesterol), and another containing unsaturated L-α-(dipalmitoleyl)-lecithin (composition 0·0175 per cent. cardiolipin, 0·2275 per cent. lecithin, and 0·3 per cent. cholesterol). These antigens had previously been found to have the same sensitivity as our standard Kolmer antigen (Tonks and Allen, 1953, 1955).

Stability and reproducibility studies were conducted along similar lines to those made with the VDRL test.

TABLE III
KOLMER TEST
ANTIGENS CONTAINING BAER'S L-α-(DIOLEYL)-LECITHIN COMPARED WITH STANDARD ANTIGEN

Antigen No.	Composition (g./100 ml.)			Ratio of Cardiolipin to Lecithin	Comparison with Standard Antigen
	Cardiolipin	Lecithin	Cholesterol		
9	0·0175	0·095	0·3	1: 5·4	More reactive
11	0·0175	0·150	0·3	1: 8·6	More reactive
10	0·0175	0·175	0·3	1:10·0	More reactive
17	0·0175	0·200	0·3	1:11·4	More reactive
21	0·0175	0·2275	0·3	1:13·0	Same
20	0·0175	0·235	0·3	1:13·4	Very slightly less reactive
16	0·0175	0·245	0·3	1:14·0	Slightly less reactive
19	0·0175	0·290	0·3	1:16·6	Less reactive

Generally speaking, in comparing antigens, tests were performed on at least 3 days with individual sera and dilutions of pooled positive sera giving negative, weakly positive, or positive reactions with our standard antigens. Some idea of their specificity was obtained by examining a group of sera which had previously been found to be negative with several tests.

EXPERIMENTAL RESULTS

(A) L-α-(Dioleyl)-Lecithin as an Antigen Component

In these studies, 21 lots of L-α-(dioleyl)-lecithin obtained from Dr. Baer were tested. As stated previously, many of them gave antigens containing

precipitates. Fortunately, however, no difficulties in this regard were experienced with Lots 2 and 3, with which most of the work reported here was carried out. Subsequent lots, purified in different ways by Drs. Baer and Buchnea, gave varying amounts of precipitate, until finally a pure material behaving very similarly to Lots 2 and 3, but giving slightly more sensitive antigens, was obtained (No. 20). Lot 21, prepared in the same way as No. 20, gave the same results as the latter. All preparations gave antigens that possessed serological activity.

(1) **VDRL Test.**—The results obtained with antigens containing L-α-(dioleyl)-lecithin in varying amounts, and standard VDRL antigen, are summarized in Table I.

Antigen No. 8, of composition 0·03 per cent. cardiolipin, 0·45 per cent. lecithin, and 0·9 per cent. cholesterol, was selected as the most satisfactory. Fine particles were obtained in the saline suspensions and when these were mixed in the usual manner with negative sera. However, the particles were very slightly larger than those obtained with the regular VDRL antigen. With positive sera, typical clumping occurred. No positive reactions were obtained with 35 negative sera which were tested with Antigen No. 8.

In Table II are given the results obtained in the antigen suspension stability studies which were carried out in duplicate. The reactivity of each suspension remained almost exactly the same over the 24-hour test period. Heating similar suspensions at 56°C. for 5 minutes immediately after preparation had little effect.

No change in reactivity was detected in antigen which was stored at room temperature in the dark for 8 months. The stock alcohol solution of Lot 3 lecithin was found to be unchanged after similar storage for 12 months. Also, the solid material (Lot 3) showed no change after 6 months storage in a desiccator at room temperature under the usual laboratory lighting conditions.

Antigens of composition No. 8 from Lots 20 and 21 L-α-(dioleyl)-lecithin (final product) proved to be identical in performance to each other and very similar to corresponding antigens containing Lots 2 and 3, although they were very slightly more sensitive than the latter. Many of the other lots gave satisfactory antigens after the precipitates which formed were removed by centrifugation.

(2) **Kolmer Test.**—The results obtained with the various antigen compositions are summarized in Table III. Titres of 1:300 were used throughout.

Antigen No. 21 of composition 0·0175 per cent. cardiolipin, 0·2275 per cent. L-α-(dioleyl)-lecithin, and 0·3 per cent. cholesterol gave results which were close to those obtained with our standard Kolmer antigen. No positive reactions occurred with any of 48 negative sera which were tested with Antigen No. 21.

In reproducibility and stability studies, similar results were obtained to those described above for the VDRL test.

(B) L-α-(Dioleyl)-Lecithin Compared with Other Lecithins

Several apparently pure, individual lecithins have now been used by us in place of Pangborn's lecithin in cardiolipin antigens. The concentrations necessary to give antigens of standard sensitivity have been determined for each lecithin. In Table IV are given the composition of antigens containing three of these lecithins and Pangborn's lecithin. Parallel tests were carried out with them on several days with a large number of individual and control sera; the results verified that they have similar sensitivities.

It can be seen from Table IV that the antigens contain different concentrations of the lecithins. In VDRL antigens, less of Pangborn's lecithin is required than of the others; next in effectiveness per unit weight is Baer's saturated lecithin, followed by his unsaturated and then Hanahan's yeast lecithin. With the Kolmer antigens, the order of Baer's

TABLE IV

COMPOSITION OF ANTIGENS OF STANDARD SENSITIVITY CONTAINING DIFFERENT LECITHINS

Lecithin Component	VDRL Antigens				Kolmer Antigens			
	Optimum Composition (g./100 ml.)			Ratio of Cardiolipin to Lecithin	Optimum Composition (g./100 ml.)			Ratio of Cardiolipin to Lecithin
	Cardiolipin	Lecithin	Cholesterol		Cardiolipin	Lecithin	Cholesterol	
Pangborn Lecithin	0·03	0·232	0·9	1:7·75	0·0175	0·200	0·3	1:11
Baer's Synthetic Saturated L-α-(dimyristoyl)-Lecithin	0·03	0·300	0·9	1:10	0·0175	0·122	0·3	1:7
Baer's Synthetic Unsaturated L-α-(dioleyl)-Lecithin	0·03	0·450	0·9	1:15	0·0175	0·228	0·3	1:13
Hanahan's Unsaturated L-α-(dipalmitoleyl)-Lecithin from Yeast	0·02	0·475	0·9	1:23·75	0·0175	0·262	0·3	1:15

TABLE V

COMPARISON OF PROPERTIES OF VARIOUS LECITHINS AND AN "IDEAL" LECITHIN, CONSIDERED AS ANTIGEN COMPONENTS

Property		"Ideal" Lecithin	Hanahan's Unsaturated Lecithin	Baer's Unsaturated Lecithin	Baer's Saturated Lecithin	Pangborn Lecithin
Formula		—	CH_2O–CO–R | CHO-CO-R | O CH_2–OP–O–choline \ OH R = $C_{15}H_{29}$ (dipalmitoleyl)	Same but R = $C_{17}H_{33}$ (dioleyl)	Same but R = $C_{13}H_{27}$ (dimyristoyl)	Mixture, chiefly unsaturated
Preparation	Method ..	—	From yeast. Solvent ext'n. and pptation + chromatography	Synthetic*—from D-acetone glycerol	Synthetic*—from α, β-dimyristin	From egg yolk or beef heart. Purified by organic solvents and metal salts
	Ease ..	Simple	Relatively easy	Difficult	Difficult	Rather difficult
	Yield ..	—	10 lbs.–3·5 g.	26% yield	33% yield	12 eggs–6 g.
Purity		Pure	Good	Good	Very good	(Mixture)
Form		Powder	Soft wax	Firm wax	Powder	Soft wax
Reproducibility ..		Every lot the same	Promising	Promising	Promising	Slight variations
Stability	as Solid ..		Good	Good	Good	Unstable
	in Ethanol	Stable indefinitely	Good	Good	Good	Good
	in Antigens		Good	Good	Good	Good
Order of Effectiveness in VDRL Antigen ..		—	4th	(See Table IV) 3rd	2nd	1st
Antigen Sensitivity ..		Positive with all syphilitics	Good	Good	Good	Good
Antigen Specificity ..		Negative with non-syphilitics	Appears good	Appears good	Good	Good
Appearance of Negatives in VDRL test ..		Fine particles	Good	Sl. rough	Sl. rough	Good
Availability		Commercial quantities	Research quantities	Research quantities	Commercial quantities	Commercial quantities

* See Baer (1956). The α, β-dimyristin is prepared from D-acetone glycerol, which is also synthetic.

saturated lecithin and Pangborn lecithin is changed; the others remain in the same position.

It is interesting to compare other properties of these lecithins, keeping in mind their usefulness as antigen components. This comparison is made in Table V, and also included are the hypothetical properties of an "ideal" lecithin.

When comparing the lecithins in the laboratory, positive control serum from rabbits (Affleck and Allen, 1954) was used for part of the work. It was interesting to find that the antigens containing Baer's saturated lecithin reacted poorly with these sera, whereas antigens containing the unsaturated lecithins gave the same results as the standard antigens. This positive serum is produced in rabbits by the intravenous injection of floccules prepared by mixing human syphilitic serum and antigen suspension. The results suggest that the lecithins themselves may possess some specific activity.

DISCUSSION

Several questions arise from comparing the lecithin concentrations required to give antigens of standard sensitivity, as listed in Table IV. Why is Pangborn's lecithin more effective per unit weight than the other lecithins in the VDRL test? Why is so much more of Hanahan's lecithin required than Baer's, which is also unsaturated, to produce antigens of the same sensitivity? Why is the saturated lecithin more effective than both of these in the VDRL test when Pangborn's, which is 80 per cent. unsaturated, is the most effective of all?

The differences may be due partly to the size of the fatty acid chains in the molecules, since we have found that L-α-(dimyristoyl)-lecithin with 14 carbon atoms in the fatty acid gives better and more reactive antigens than L-α-(dipalmitoyl)-lecithin with 16 carbon atoms. However, less of Baer's dioleyl lecithin, with 18 carbon atoms in the fatty

acid, is required than Hanahan's dipalmitoleyl lecithin with 16. Testing of other lecithins with fewer carbon atoms would give further data on this point.

Possibly the unsaturated compounds of Hanahan and Baer contain traces of impurities which are not detectable by chemical tests but which affect serological reactions. No direct evidence for this has been found. Another possibility is that Pangborn's preparation, which is a mixture, may contain a lecithin that is more specific for the detection of "reagin" than the pure ones we have tested, a supposition that is supported indirectly by the fact that the saturated lecithin antigens react poorly with the "positive" rabbit serum referred to above whereas unsaturated lecithin antigens react normally; or Pangborn's lecithin may contain another type of compound in small amounts which is more effective than any of the pure compounds tested so far. These are interesting possibilities. In this connexion there should be mentioned the identification of phosphatidal choline as the major constituent of beef heart lecithin (Rapport and Alonzo, 1955).

In order to choose which is the best of these lecithins for use in antigens, we must first decide upon the properties which are important and necessary. An "ideal" lecithin would be a pure compound, solid at working temperatures and preferably a crystalline powder, easy to prepare and to reproduce exactly from batch to batch; it would be stable in its solid form, in alcohol solution, and in antigens at all reasonable temperatures; it would be completely soluble in absolute alcohol in amounts suitable for the preparation of antigens, and would give antigens of the desired sensitivity and specificity. With such a material repeated standardizations would be eliminated since the correct concentration of the lecithin component (and therefore the desired sensitivity) could be obtained merely by an accurate weighing.

None of the pure lecithins discussed here can be considered yet to be ideal; however, they are close to being so. The unsaturated ones are waxes and are perhaps not quite as easy to handle as the saturated lecithin, which is a crystalline powder; however, this is not an important factor. Different lots of Hanahan's lecithin have thus far given slightly different results; but its purity could without doubt be improved and it is the easiest to obtain. Baer's unsaturated lecithin has no presently apparent major fault other than that of being rather difficult to make, but it must be tested further for specificity, stability, and reproducibility. The saturated lecithin is also difficult to prepare, and gives slightly rougher negative pictures than the others; however, in extended trials, antigens containing it have rated very well as to sensitivity and specificity.

Stability towards oxygen is an important factor. It is curious that the pure unsaturated lecithins of Baer and Hanahan are quite stable out of solution (Baer, Buchnea, and Newcombe, 1956), unlike Pangborn's material which must be stored in alcohol. This property of the pure materials may be due to the absence of substances (removed by chromatographic techniques or not present originally) which are believed to catalyse the rapid breakdown of lecithins by oxidation of the fatty acids at the double bonds (*e.g.*, metals such as copper and iron). The stability of Pangborn's lecithin in its solid form might be improved by further purification by chromatography.

It should be remembered that only Pangborn's lecithin has been prepared commercially* and in large lots, and that the reproducibility and purity of the other lecithins would probably be improved if this was done for them also.

Summary

(1) A pure unsaturated synthetic lecithin, L-α-(dioleyl)-lecithin, has been tested as a component in cardiolipin antigens for the VDRL microflocculation test and the Kolmer complement-fixation test for syphilis and has given very promising results.

(2) This lecithin has been compared with others with regard to its use as a component in cardiolipin antigens. The properties of an "ideal" lecithin are discussed.

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* Dimyristoyle lecithin is now also being prepared commercially.